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The Exogenous Form of Jaagsiekte Retrovirus Is Specifically Associated with a Contagious Lung Cancer of Sheep

M. PALMARINI,¹ C. COUSENS,¹ R. G. DALZIEL,² J. BAI,³ K. STEDMAN,³
J. C. DEMARTINI,³ AND J. M. SHARP^{1*}

Moredun Research Institute, Edinburgh EH17 7JH,¹ and Department of Pathology, Faculty of Veterinary Medicine, Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh EH9 1QH,² United Kingdom, and Department of Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado 80523³

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Sheep pulmonary adenomatosis ([SPA] ovine pulmonary carcinoma) is a transmissible lung cancer of sheep that has been associated etiologically with a type D- and B-related retrovirus (jaagsiekte retrovirus [JSRV]). To date it has been impossible to cultivate JSRV in vitro and therefore to demonstrate the etiology of SPA by a classical approach. In addition, the presence of 15 to 20 copies of endogenous JSRV-related sequences (*enJSRV*) has hampered studies at the molecular level. The aim of this study was to investigate whether the expression of exogenous JSRV was specifically associated with neoplasia in SPA-affected animals. Initially, we found that *enJSRV*s were transcribed in a wide variety of normal sheep tissues. Then, by sequencing part of the *gag* gene of *enJSRV* we established a *ScaI* restriction site in *gag* as a molecular marker for the exogenous form of JSRV. Restriction enzyme digestion of PCR products obtained from the amplification of cDNA from a total of 65 tissues collected from SPA-affected and unaffected control sheep revealed that the exogenous form of JSRV was exclusively and consistently present in tumor tissues and lung secretions of the affected animals. In addition, exogenous JSRV provirus was detected only in DNA from SPA tumors and not from nontumor tissues of the same animals. This study has demonstrated clearly that the exogenous form of JSRV is specifically associated with SPA tumors.

Retroviruses are an invaluable model system for understanding carcinogenesis and the regulation of normal cell growth. Studies of spontaneous retrovirus-induced animal and human lymphomas have contributed immensely to our knowledge of the onset of cancer (5), yet relatively little information is available concerning retroviral causes and mechanisms of epithelial cell neoplasms which, in humans, are much more frequent than lymphomas (39).

Sheep pulmonary adenomatosis (SPA; also known as jaagsiekte and ovine pulmonary carcinoma) represents a unique model of a naturally occurring epithelial neoplasm which is associated etiologically with a retrovirus (6, 35). Interestingly, SPA shares certain characteristics with human bronchoalveolar cell carcinoma (13, 29), which is only weakly associated with smoking (10, 16, 21) and which has dramatically increased in prevalence since 1955 and now represents 24% of lung cancers (3).

Several lines of evidence implicate retroviral involvement in the etiology of SPA. SPA has been reproduced experimentally by inoculating sheep with cell-free lung secretions (lung fluid) or tumor extracts from SPA-affected animals (7, 24, 36, 42). These materials have been shown consistently to contain a retrovirus with morphological, biochemical, and immunological properties similar to type D and B retroviruses (20, 30, 32, 38). This virus, known as jaagsiekte retrovirus (JSRV; also known as SPA retrovirus and ovine pulmonary carcinoma retrovirus), exhibits the typical genomic organization of a replication-competent retrovirus with long terminal repeat (LTR), *gag*, *pro*, *pol*, and *env* regions and no apparent sequences commonly associated with transformation (43). Despite the clear

association between JSRV and pulmonary adenomatosis, classical approaches to demonstrate an etiological role for this virus have been hampered by the lack of a cell culture system to propagate JSRV.

Recently, the link between JSRV and SPA was strengthened further by the demonstration that lung and transformed pulmonary epithelial cells of SPA-affected sheep are major sites of virus replication. JSRV particles or antigens have never been detected in tissues of unaffected control sheep (27). In addition, the presence of 15 to 20 copies of endogenous JSRV-related sequences (*enJSRV*) in the normal sheep genome has hampered analysis at the molecular level (19, 43). No information is available about *enJSRV* sequences and their degree of similarity with the exogenous JSRV. Therefore, the possibility cannot be excluded that expression of the exogenous virus in neoplastic cells is the result of the reactivation of an *enJSRV* as a downstream event of the neoplasia.

The aim of this study was to investigate expression of JSRV in tumor tissues and whether it is specifically associated with neoplasia. Initially, the expression of JSRV-related sequences in tissues of SPA-affected and unaffected control sheep was determined by reverse transcription-PCR (RT-PCR). Differentiation between exogenous and endogenous RNA species was evaluated by sequencing and/or restriction enzyme digestion of PCR products obtained from viral pellets and tumors of SPA-affected sheep and tissues of unaffected sheep. Comparison between tumor and nontumor genomic DNA collected from the same SPA-affected animals was then performed in order to determine whether the exogenous proviral JSRV was detectable only in genomic DNA from tumor tissues.

MATERIALS AND METHODS

Sources and preparation of samples. In this study, SPA-affected sheep were defined as animals which showed typical clinical signs, particularly the produc-

* Corresponding author. Phone: 44 131 6643262. Fax: 44 131 6648001. Electronic mail address: sharm@scot-moredun.agric-res.inst.ac.uk.

TABLE 1. Tissues examined by RT-PCR (*gag*) followed by *ScaI* digestion in SPA-affected and unaffected control sheep

Tissue	SPA ^a		SPA ^b	
	PCR (no. tested/no. positive)	<i>ScaI</i> digestion (no. tested/no. sensitive)	PCR (no. tested/no. positive)	<i>ScaI</i> digestion (no. tested/no. sensitive)
Lung fluid	16/16	16/16		
Tumor/lung	5/5	5/5	4/4	4/0
Kidney	3/3	3/0	3/3	3/0
Thymus	2/2	2/0	3/3	3/0
Bone marrow	3/3	3/0	3/3	3/0
Spleen	2/2	2/0	3/3	3/0
Mediastinal lymph node	5/5	5/3	3/3	3/0
Leukocytes	5/5	5/0	5/5	5/0
Total	41/41	41/24	24/24	24/0

^a SPA-affected sheep.^b Unaffected control sheep.

tion of abundant seromucoid fluid (lung fluid) from the nostrils when the rear limbs were elevated above the head. Diagnosis was confirmed by macroscopic and histological examination of the lungs. Both naturally SPA-affected animals and lambs infected by intratracheal inoculation of concentrated lung fluid, as previously described (36), were used in this study. In addition, age- and breed-matched controls were employed.

A total of 16 lung fluid samples were collected during clinical examinations of SPA-affected sheep and processed as already described (38). Briefly, 5 to 10 ml of lung fluid was filtered through a double layer of gauze and clarified by centrifugation at $10,000 \times g$ for 1 h at 4°C. The supernatant was then centrifuged at $100,000 \times g$ through a double layer of glycerol (25 and 50% [vol/vol]) for 1 h at 4°C, and the resultant pellet was resuspended in 500 µl of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7], 0.5% sarcosyl, 0.1 M β-mercaptoethanol) and stored at -20°C until use. Leukocytes were obtained from 10 blood samples in which the erythrocytes had been lysed. A total of 10^7 cells for each sample were resuspended in 500 µl of solution D and stored at -20°C.

Tissues from five lung tumors, four normal lungs, eight mediastinal lymph nodes, five thymus glands, six bone marrow samples, five spleens, and six kidneys of both SPA-affected and unaffected sheep were collected at postmortem examination (Table 1) and snap-frozen in liquid nitrogen before storage at -70°C. Dog lung tissues were treated as the sheep tissues and used as negative controls. An equine dermis cell line and primary fetal lamb lung and kidney cells were employed as further controls in this study. Cells (10^7) were resuspended in 500 µl of solution D and stored at -20°C.

RNA extraction. Total RNA from ultraspeed pellets of lung fluid (2.5 to 5.0 ml), sheep tissues, and dog lung tissues (0.2 to 0.5 g), leukocytes, equine dermis cells, and fetal lamb lung and kidney cells (10^7 cells) was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (4). Each RNA sample was treated with 10 U of DNase I, RNase free (Boehringer), as suggested by the manufacturer.

DNA extraction. High-molecular-weight genomic DNA was extracted by established procedures (34) from lung tumor and kidney samples from four of the five SPA-affected animals and from lung and kidney tissues from three of the four unaffected controls. DNA was also isolated from primary cultures of normal sheep skin fibroblasts for analysis of the LTRs (see below). Briefly, pulverized tissue or cell pellets were lysed in TES buffer (10 mM Tris, 0.1 M EDTA, 0.5% sodium dodecyl sulfate [pH 8]) followed by digestion with RNase A (Sigma; 20 µg/ml) for an hour at 37°C with gentle mixing, then 100 µl (20 µg/ml) of proteinase K was added and incubated at 60°C overnight. Separate phenol and chloroform-isoamyl alcohol (24:1) extractions were followed by isopropanol precipitation. High-molecular-weight DNA was harvested by spooling it onto glass pipettes and was washed twice with 70% ethanol on ice, dried, resuspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 8]), and stored at 4°C.

RT-PCR (*gag*). Synthesis of cDNA was carried out in 25-µl reaction volumes as follows. Total RNA (2 µg from tissues, leukocytes, and cell culture samples or total RNA obtained from ultraspeed pellets of 5 to 10 ml of lung fluid) was diluted in 13.5 µl of diethyl pyrocarbonate-treated distilled water. RNA was denatured at 65°C for 10 min and then cooled on ice. One microliter (40 U) of rRNasin (Promega)-2 µl of 25 mM each deoxynucleoside triphosphate (dNTP)-2.5 µl of 100 mM dithiothreitol-2.5 µl (A_{260} , 62.5 U/ml [Boehringer]) of random hexamers-2.5 µl of 10× reaction buffer (500 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$)-1 µl of Stratascript reverse transcriptase (50 U; Stratagene) was added. The addition of the reverse transcriptase was omitted in control samples to check for DNA contamination of the RNA preparations. The reaction mixture was incubated at 37°C for 1 h, and then the enzyme was inactivated by incubation at 94°C for 3 min.

Amplification of the resultant cDNA was carried out by PCR. Primers were

designed on the basis of the complete sequence of JSRV (43) and the pCA1 sequence of *gag* (19). Primers P1 and P2 (Fig. 1A) spanned a 229-bp region internal to the *gag* gene (position 1598 to 1826 of JSRV) (43) (sense [P1], 5'-GCTGCTTTAGACCTTATCGAAA-3'; antisense [P2], 5'-ATACTGCAG CYCGATGGCCAG-3'). Three microliters of cDNA was added to 47 µl of 1× PCR buffer (1.25 U of *Taq* polymerase [Boehringer], 2.75 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 200 µM each dNTP, 6.25 pmol of each primer). PCR cycles employed were 94°C for 1 min and 35 cycles of 94°C for 45 s, 57°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 2 min in a Perkin-Elmer GenAmp 2400 thermal cycler.

PCR was optimized for the detection of 1 to 10 copies of target DNA, using plasmid pJS382 (courtesy of G. Querat) containing part of the JSRV *gag* gene (nucleotide 953 to 3029) as template. Amplified products were detected by electrophoresing 25-µl aliquots through a 2% agarose gel in 1× TBE buffer in the presence of 0.5 µg of ethidium bromide per ml.

The products of five independent PCR-*gag* procedures with kidney cDNA from an unaffected sheep were pooled before being cloned in pGEM-T (Pro-

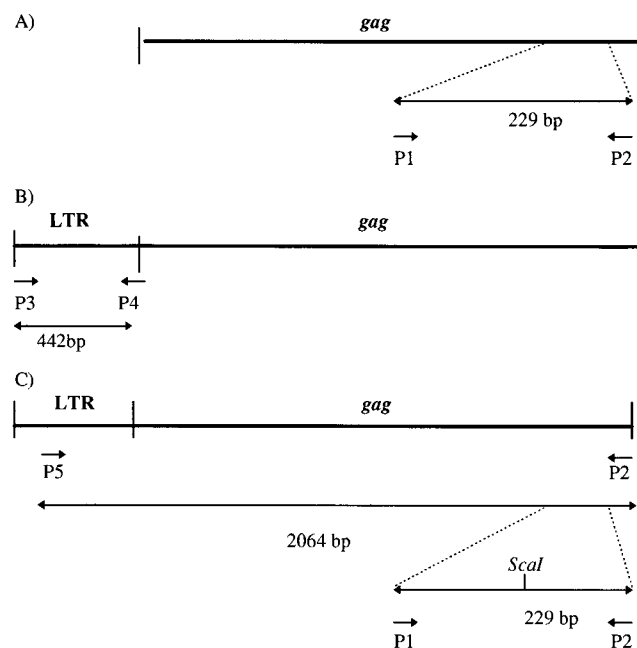


FIG. 1. PCR strategy and localization of primers employed in this study. (A) RT-PCR (*gag*), employing primers P1 and P2, amplified a product of 229 bp. (B) PCR for the amplification of endogenous LTRs employed primers P3 and P4 and generated products of 442 to 444 bp. (C) Heminested LTR-*gag* PCR. The first round of amplification used primer P5 (internal to the LTR) and *gag* primer P2. One microliter of this reaction mixture was used in the second round of amplification, which used primers P1 and P2.

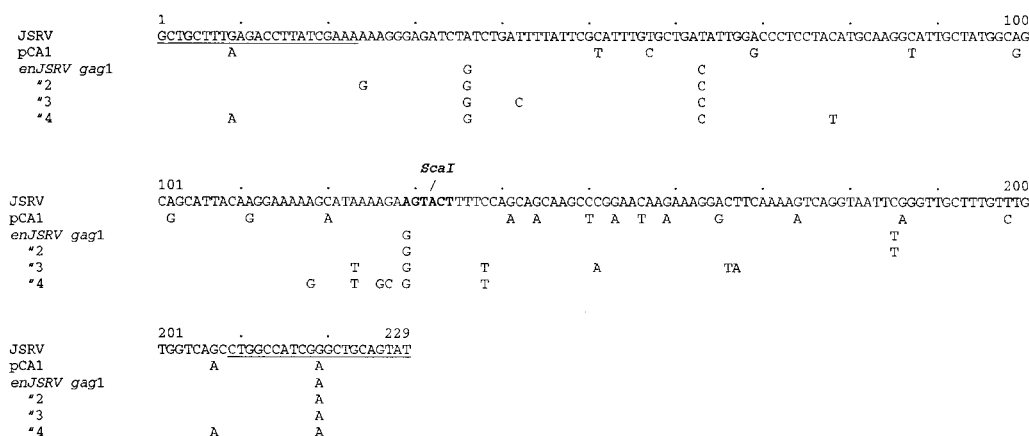


FIG. 2. Comparison of *enJSRV gag* sequences and exogenous pCA1 (19) with JSRV (43). Indicated are base differences with JSRV. Primers used are underlined, while the boldface bases indicate the exogenous-specific *ScaI* site.

mega), following the manufacturer's protocol. Seven clones were selected and sequenced with the T7 sequencing kit (Pharmacia), as recommended by the manufacturer.

Restriction enzyme digestion. Twenty-five microliters of the PCR-gag product was digested with 13 U of *ScaI* (Boehringer), employing the appropriate incubation buffer supplied by the manufacturer (PCR buffer was ignored), at 37°C for 2 to 4 h. The digestion products were analyzed alongside the corresponding mock-digested PCR products, by electrophoresis in a 2% agarose gel stained with ethidium bromide.

Amplification and sequencing of endogenous LTRs. One hundred nanograms of high-molecular-weight genomic DNA isolated from sheep skin fibroblasts was used for PCR amplification of endogenous LTRs (PCR-LTR). Primers (Fig. 1B) were designed at the 5' end of U3 (sense [P3], CTGCGGGGGACGAC, position 7179 to 7193 [43]) and at the 3' end of U5 (antisense [P4], CTGCGGCGAC CAGCA, position 111 to 125 [43]). The buffer employed was 10 mM Tris (pH 8.9), 50 mM KCl, 2 mM MgCl₂, with 200 μM each dNTP, 100 ng of primers, and 1 U of *Taq* (Boehringer). PCR cycles employed were an initial step of 94°C for 2 min and then 40 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, with a final extension of 72°C for 2.5 min.

The products of three independent PCRs were pooled and then cloned in the TA cloning system (Invitrogen), according to the manufacturer's instructions. Three resultant clones were sequenced by the dideoxy chain termination method, employing a Li-Cor automated sequencer.

Heminested LTR-gag PCR. In the first round of amplification, 250 to 500 ng of tumor and kidney high-molecular-weight DNA was amplified by PCR, employing the Expand Long Template PCR system (Boehringer), which uses a mixture of *Taq* and *Pwo* DNA polymerases. The sense primer (P5, TGGGAGCTCTTTG GCAAAGCC, nucleotide position 7210 to 7224 [43]) was designed on the basis of nucleotide sequence differences between the endogenous LTR sequences (1) and the JSRV sequence (43). Primer P2 was employed as antisense primer (Fig. 1C). Reactions were carried out in a 50-μl volume containing 50 mM Tris-HCl (pH 9.2), 160 mM (NH₄)₂SO₄, 2.25 mM MgCl₂, 2% (vol/vol) dimethyl sulfoxide, 0.1% (vol/vol) Tween 20, 500 μM each dNTP, 300 nM each primer, template DNA, and 2.625 U of enzyme mix. Cycles were as follows: 1 cycle at 92°C for 2 min; 30 cycles at 92°C for 10 s, 61°C for 30 s, and 68°C for 90 s (plus a progressive increment of 20 s from cycle 11 onward); and a final extension of 68°C for 7 min.

One microliter of the product of the first round of amplification was used in the second round of PCR which was the same as the PCR-gag procedure described above. Half the product of the second-stage PCR was digested with *ScaI* as described above.

Products of the first and second rounds of heminested PCR were visualized by electrophoresis in 1 and 2% agarose gels, respectively.

Nucleotide sequence accession numbers. The nucleotide sequences of the PCR clones of *enJSRV gag* fragments and LTRs are deposited in GenBank under accession numbers Z49800, X92180, X92181, X92182 (*enJSRV gag1* to -4) and Z66531, Z66532, Z66533 (*enJSRV* LTRs 1 to 3).

RESULTS

Detection of *enJSRV gag* transcripts and differentiation between endogenous and exogenous transcripts. RT-PCR (*gag*), using the primer pair P1 and P2, was used to examine respiratory and other tissues from both SPA-affected and unaffected sheep for the presence of JSRV *gag* transcripts. The results are summarized in Table 1.

A product of the expected size (229 bp) was detected in all 65 sheep tissue samples tested. Primary cultures of fetal lamb and kidney cells produced the same visible amplicon, whereas dog lung tissues and an equine dermis cell line were consistently negative. To exclude DNA contamination in the total RNA preparations, the same samples were tested in parallel, omitting reverse transcriptase from the reaction mix; no signal was obtained from any of these samples.

The specificity of the amplicons was confirmed by Southern hybridization with an oligonucleotide probe from a region of JSRV *gag* internal to the PCR product (data not shown).

Previous studies have shown consistently that lung fluid and lung tumor tissues from SPA-affected sheep contain JSRV particles and antigens (18, 20, 27, 38); therefore, it may be assumed that the RNA detected in these materials is of exogenous origin, while the RNA detected from tissues of unaffected control animals is transcribed from endogenous sequences. RT-PCR using primers P1 and P2 therefore demonstrated that transcripts of endogenous origin are widely expressed in sheep tissues.

To identify possible differences between endogenous and exogenous RNA, the RT-PCR products obtained from kidneys of an unaffected control animal were cloned and sequenced. Seven clones were sequenced, and four different sequences (*enJSRV gag1* to -4) were obtained.

The four *enJSRV* sequences were compared with each other and with the published exogenous sequences of JSRV (43) (South African isolate) and pCA1 (19) (Peruvian isolate) by computer analysis using the GCG Pileup program (14) (Fig. 2). The South African and the Peruvian isolates were 90% similar in the stretch of sequence considered. The *enJSRV* clones were highly homologous to each other (95 to 99% similarity), 95 to 98% similar to the South African isolate, and 85 to 88% similar to the Peruvian isolate. Comparison of the restriction maps of the six sequences revealed a *ScaI* restriction site in JSRV and pCA1 that was absent in all the *enJSRV* clones. To confirm this observation, RT-PCR products from 16 lung fluid samples and 44 nontumor tissues were digested with *ScaI*. Successful digestion, indicated by the presence of two smaller fragments of 131 and 98 bp, was observed with all 16 lung fluid samples, 5 of 5 tumors, and faintly in 3 of 5 mediastinal lymph nodes from SPA-affected sheep. No *ScaI*-sensitive product was observed with any other tissue (Fig. 3).

These findings confirm the complete correlation between the *ScaI* site and the exogenous virus.

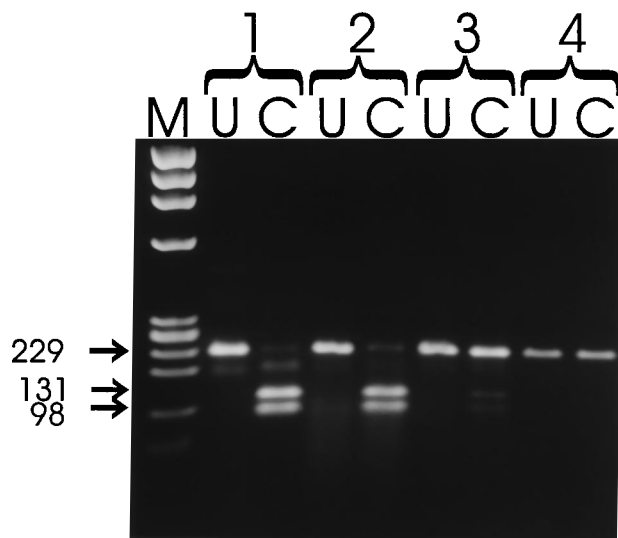


FIG. 3. Typical results of RT-PCR, followed by *ScaI* digestion, of total RNA from SPA-affected sheep tissues. Samples: 1, lung fluid; 2, lung tumor; 3, mediastinal lymph nodes; 4, kidney (samples 1 to 4 were collected from the same animal). Lanes: U, mock-digested PCR products; C, PCR product digested with *ScaI*; M, molecular weight marker IX (Boehringer).

Amplification and sequencing of endogenous LTRs. PCR products of approximately 450 bp were obtained from the amplification of DNA from cultured sheep primary skin fibroblast cultures by employing primers P3 and P4. Products were cloned as described in Materials and Methods, and three clones were completely sequenced (Fig. 4).

The *enJSRV* LTRs appeared to be longer (442 to 445 bp) than the published exogenous LTR (397 bp) which showed two large deletions of 30 and 16 bp at positions 198 and

261, respectively (nucleotide positions as in Fig. 4). The nucleotide sequences of the three *enJSRV* LTRs were 83 to 90% identical and were 69 to 76% similar to the JSRV LTR sequence (43).

Detection of exogenous JSRV in tumors of SPA-affected sheep. PCR products obtained by direct amplification of tumor genomic DNA with primers P1 and P2 followed by *ScaI* digestion did not show any cut product by electrophoresis in an agarose gel stained with ethidium bromide, and hybridization of Southern blots with an internal probe did not resolve this issue (data not presented). The failure to detect *ScaI*-sensitive sequences at the DNA level may be due to the high proportion of endogenous template compared with exogenous template, resulting in a small proportion of *ScaI*-sensitive product. Theoretically, there may be 20 endogenous copies compared with possibly only 1 exogenous copy per neoplastic cell. Moreover, neoplastic cells are approximately only 40 to 50% of the total number of cells present in the neoplastic tissue, therefore increasing the ratio of endogenous to exogenous sequences.

To preferentially amplify exogenous proviral DNA sequences (with the *ScaI* restriction site) we designed a hemi-nested PCR. The first round of amplification used a sense primer (P5) based on an area of sequence divergence between endogenous LTR clones and the exogenous LTR sequence (JSRV) and the antisense primer (P2) of the RT-PCR (*gag*). A product of about 2 kb was visible in an ethidium bromide agarose gel from three of four SPA tumor DNAs, while no signal was obtained in four of four kidney DNAs of the same animals or from lung and kidney tissues of three unaffected control animals. A second round of amplification, using primers of the RT-PCR (*gag*) followed by *ScaI* digestion, enabled the detection of *ScaI*-sensitive sequences in all four tumors examined, while only spurious bands were detected in nontumor samples of SPA-affected or nonaffected sheep (Fig. 5).

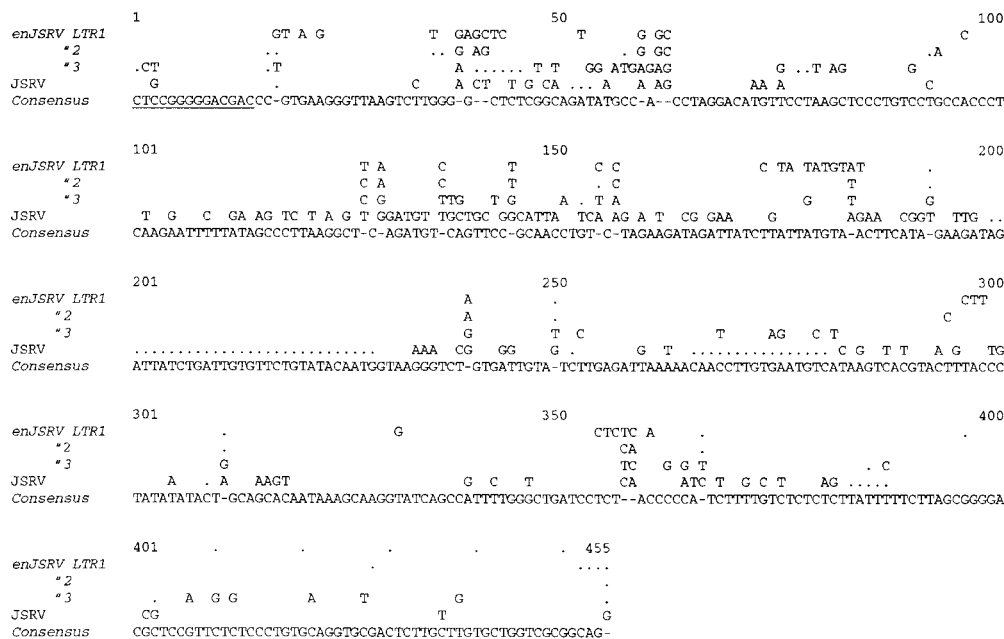


FIG. 4. Sequence alignment of *enJSRV* LTR clones with the JSRV LTR (43). Letters indicate differences with the consensus sequence. A period (.) indicates deletions. Primers used to obtain *enJSRV* clones are underlined.

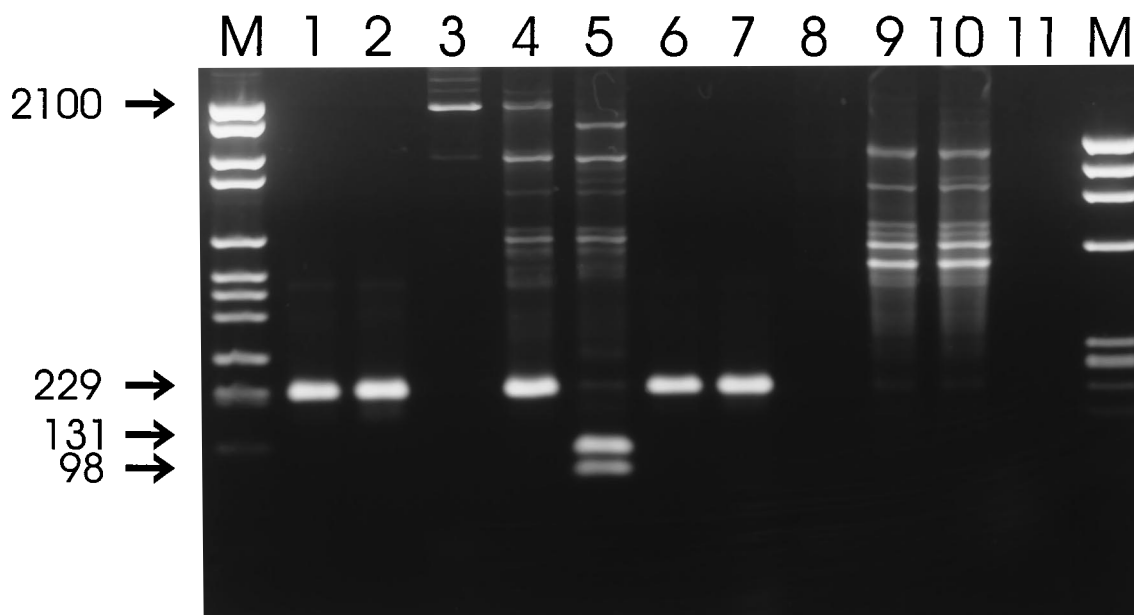


FIG. 5. Detection of exogenous JSRV sequences in tumor and kidney DNAs of the same SPA-affected sheep. Lanes 1 to 5 represent experiments performed with lung tumor genomic DNA. Lanes: 1, 500 ng of lung tumor genomic DNA amplified directly by PCR-*gag*, employing primers P1 and P2; 2, PCR product shown in lane 1 cut with *ScaI*; 3, first round of amplification of heminested LTR-*gag* PCR, employing primers P5 and P2; 4, second round of amplification, employing primers P1 and P2, of 1 μ l of the PCR product shown in lane 3; 5, PCR product of lane 4 cut with *ScaI*. Lanes 6 to 10 represent experiments performed with kidney genomic DNA. Lanes: 6, 500 ng of kidney genomic DNA amplified directly by PCR-*gag*, employing primers P1 and P2; 7, PCR product of lane 6 cut with *ScaI*; 8, first round of amplification of heminested LTR-*gag* PCR, employing primers P5 and P2; 9, second round of amplification, employing primers P1 and P2; 10, PCR product shown in lane 10 cut with *ScaI*. Lane 11 contains a negative control, lanes M contain Boehringer molecular weight markers VI (left) and IX (right).

DISCUSSION

This study has provided compelling lines of evidence which collectively demonstrate a specific association between the exogenous forms of JSRV and SPA.

It was shown clearly that the exogenous form of JSRV was distinct from endogenous JSRV transcripts as identified by the presence of a *ScaI* restriction site in *gag* (nucleotide position 1729 [43]). Furthermore, this site was conserved in strains of exogenous JSRV obtained from geographically dispersed countries, supporting data linking these viruses to each other and to SPA (18, 20, 27, 28, 33, 38).

Exogenous JSRV RNA was found only in tumors, lung fluid, and draining lymph nodes of SPA-affected sheep, which is in agreement with results of an earlier immunological study, which concluded that epithelial tumor cells are major sites of replication for JSRV (27). Lung fluid and tumor tissues from SPA-affected sheep are the only materials that have been used successfully to reproduce the tumor experimentally in sheep and goats (7, 24, 36, 37, 42). These findings, therefore, point to exogenous JSRV as a strong candidate for the etiological agent of SPA, although they do not rule out possibilities such as reactivation of an *enJSRV* as a downstream event of neoplasia. However, the demonstration of exogenous JSRV provirus only in tumor DNA provides irrefutable evidence that this virus is specifically associated with neoplasia. Endogenous JSRV transcripts were present in a wide variety of tissues from both SPA-affected and unaffected sheep, although downstream translation may be very low or absent, because CA protein was not detected in nontumor tissues (27).

Nevertheless, the presence of transcriptionally active *enJSRVs* raises a question about their potential role in SPA. In mice, chickens, and cats, endogenous retroviruses have been demonstrated to be involved in recombinational events leading to the generation of oncogenic retroviruses or viruses with a

broader host range (2, 12, 15, 25, 41). The possibility of recombinational events between the exogenous form of JSRV and its endogenous homologs cannot be ruled out. To clarify this point, further characterization of endogenous and exogenous JSRV sequences will be essential as limited sequence data are available (19, 43).

Comparison of LTR sequences between JSRV and three *enJSRV* clones derived from sheep skin fibroblasts revealed differences in putative enhancer sites. A putative NF-1 motif in exogenous JSRV LTR (nucleotides 7220 to 7223), which overlaps the exogenous-specific PCR primer P5, is altered and/or lost in the endogenous LTRs, and two tandemly repeated sequences (positions 7347 to 7365 and 7366 to 7384 in the JSRV sequence [43]) appear to be lost by means of an insertion of 30 nucleotides in the endogenous U3. Differences in the nucleotide sequences in these regions could potentially diminish the LTR function (8, 9, 11, 12, 17, 22, 23, 26, 31, 40) and thus reduce the pathogenicity of *enJSRVs* compared with the exogenous counterpart, as demonstrated in other retroviral systems.

An interesting finding of this study was the detection of exogenous JSRV RNA in three of five mediastinal lymph nodes of SPA-affected sheep. Previous immunological studies detected JSRV particles in only 1 of 12 regional lymph nodes of SPA-affected sheep (27). The detection of the exogenous JSRV in the majority of regional lymph nodes by RT-PCR may reflect the presence of metastases, although these were not detected by histological examination (data not shown), or may reflect the presence of the virus in the lymph drained by the regional lymph nodes at levels below the detection limit of the immunological techniques. Because of the lack of data on the immune response to JSRV, we cannot determine whether the presence of JSRV in the lymph nodes is related to an active immune response to the virus.

To date, JSRV has not been isolated *in vitro*, and therefore classical approaches to demonstrate the etiology of SPA have been impossible. However, the results reported in this communication show that exogenous JSRV is consistently and exclusively demonstrated in tumors and lung fluids, which have been used as inocula to induce SPA on numerous occasions (7, 24, 36, 42).

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